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ON THE CYTOLOGY AND LIFE HISTORY OF THE AMOEBAE *

WITH DESCRIPTIONS OF TWO NEW SPECIES

BY R. W. GLASER

INTRODUCTION

It was shown by Graybill and Smith (1920) and Smith and Graybill (1920) that blackhead in turkeys and chickens could be experimentally produced by feeding the embryonated eggs of the nematode, *Heterakis papillosa*. They had frequently encountered protozoa in the cultures in which the ova were developing, and it was considered advisable to determine whether or not these organisms represented some stage of the protozoon responsible for the disease. These results led the writer to make a careful study of the protozoan fauna encountered in Heterakis, as well as in the embryonated egg cultures. These investigations disclosed nothing of importance concerning blackhead, but the amoebae studied revealed a number of new and interesting cytological and life history characters.

The best method for obtaining the amoebae under discussion consists in removing adult Heterakis from the ceca of a variety of healthy chickens. The worms are thoroughly washed in sterile physiological salt solution, and sedimented several times. They are then cut up and the material incubated in physiological salt solution for fifteen to thirty days at room temperature. During the summer of 1920, nineteen cultures were thus prepared from different lots of chickens.

On examining incubated Heterakis cultures in from two to three weeks, it was found that besides worm fragments, embryonated Heterakis ova, and bacteria, the cultures nearly always contained the trophozoites and cysts of amoebae. Two species were found in the salt solution cultures either together in the same culture or separately in different cultures. The amoebae were easily recognized, particularly the cysts resting on the bottom of the Petri dish, but when the writer was in doubt, he placed a worm fragment under the microscope. Such a fragment usually proved to contain many amoebae which probably fed upon the countless bacteria found within the dead worm tissues. The two forms were the only amoebae which developed within the salt solution cultures. By the use of the methods of Frosch (1897) and Walker (1908), the amoebae were isolated and grown on Musgrave and Clegg's medium, 0.7 of normal alkaline, in association with cultures of *Staphylococcus pyogenes albus*.

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For the study of some of the stages the writer found Walker's hanging plate method indispensable, but found and Walker also noted that the amobae will usually not pass through their complete life cycle on the hanging plate but take the short cut from cyst to cyst merely through the trophozoite stage. In order to obtain all of the stages, one must also resort to the Petri plate cultures.

The various stages that were fixed and stained were always compared with the living material of similar stages that grew on the hanging plate cultures. One can obtain a much more accurate picture of the organisms from the hanging plate cultures than by removing some of the organisms from Petri plate cultures to some liquid medium like Ringer's, Locke's, or physiological sodium chloride solution. Should the latter procedure be necessary, one must be careful to use a solution strictly isotonic to the liquid constituents of the protoplasm, otherwise one produces certain deformations of the organisms, manifested by plasmolysis or extensive grosser granulations and other cytologic structural changes that do not normally exist.

When certain stages fail to develop on the hanging plate, it is necessary to consult the Petri plate cultures and examine the organisms in some carefully balanced non-toxic solution, but the best results are obtained by removing the stages from the Petri culture and by immersing them immediately in some fixative. Fixation was accomplished either by means of Zenker's or Schaudinn's solutions. Mallory's chloride of iron hematoxylin was used extensively after Zenker's fixation, but some stages of the amoebae were better elucidated by means of Delafield's or Heidenhain's iron hematoxylin after Schaudinn's corrosive sublimate alcohol mixture.

Vital staining (methylene blue and especially neutral red 1 : 3,000 to 1 : 10,000) was used during the studies on cysts and the method of sporulation. This method cleared up much that remained obscure when fixing and other staining methods were used.

It seems advisable to tentatively place the two organisms studied by the writer in the genus Amoeba rather than in the genus Entamoeba for reasons which will become apparent later. However, it might be well to state that no evidence was found to support the view that the organisms to be described multiply or pass through any stages within the intestines or ceca of chickens. Recently Tyzzer (1920) described two true host inhabiting forms in chickens and turkeys. He did not report stages of the organisms described in this article, so it seems probable that the amoebae here described are what are known as intestinal passers, possibly derived from the soil, and remain in the encysted stage until eliminated with the excrement.

Amoeba commutabilis sp. nov.

This amoeba when cultivated on the hanging plates (sealed depression slide cultures) usually presents only the simple amoeboid and cyst stages. If the medium on the cover slip is inoculated with cysts and some food bacteria *(Staph. pyogenes albus),* the trophozoites appear in 48 hours at room temperature or sooner at 32° C. The trophozoites are exceedingly sluggish. The cytoplasm is clear, faint and finely granular. There is no distinct differentiation between endo- and ectoplasm except when the pseudopodia form. The formation of the pseudopodia is eruptive, and when they erupt they often run along the periphery of the amoeba in a wave-like motion. There seems to be no pronounced flowing of the endoplasm. Sometimes the pseudopodial processes are quite pointed, but usually blunt. They are always rather short. A few food vacuoles are present containing bacteria during the feeding phase. A small contractile vacuole is present. The nucleus is clear and faint with the chromatin concentrated within the karyosome during the feeding and growing phase. Plate I, figs. 1, 2, 4 and 5 represent living amoebae of the growing and feeding phase. Figures 1 and 2 represent amoebae examined in isotonic solution and showing no clear differentiation between endo- and ectoplasm. The ectoplasm in these cases appears granular. Figures 4 and 5 represent amoebae examined on depression slide cultures showing differentiation between endoplasm and pseudopodial ectoplasm. When conditions become unfavorable on the depression slides, the amoebae first become quiescent, then free themselves of waste products, assume a round form and encyst (Fig. 6). Encystment on the depression slides occurs in four or five days. The early cysts still show the chromatin concentration represented by the karyosome which is surrounded by a clear, distinct, but chromatin free nucleus. The cysts show only one wall when examined on the depression slides or in a strictly isotonic solution in which no plasmolysis occurs. The resting trophozoites measure 5 to 8μ. The cysts measure 6μ.

The agamic multiplication of the trophozoites can best be followed by roughly detecting the nuclear and karyosomic changes on the depression slide, and by immediately removing the cover slip containing the agar film culture, immersing in the fixative, and staining carefully by one of the histologic methods outlined previously.

Neither a sexual phenomenon nor the suggestion of one was encountered. The trophozoites multiply by a sort of primitive mitosis known as promitosis, or the division of the karyosome. Hartmann did much work on this method of cell division, but certain important details found in this amoeba seem to differ from methods heretofore described. In the most primitive protozoa showing this form of nuclear division, Hartmann found that the karyosome contains all the chromatin and nuclear material and is submerged in a zone of nuclear sap. No definite nuclear membrane exists. In higher forms chromatin and supportive structures are found within the nucleus proper and a definite nuclear membrane appears.

During the course of the studies on *A. commutabilis,* it was found that at certain times the amoebae could be placed within the primitive group and at times within the modified group. At a certain stage the karyosome contains all the chromatin and is situated in clear nuclear sap containing no supportive structures. At another stage the karyosome gives off chromatic material (chromidia) into the nucleus or the karyosome may disappear entirely, all the chromatic material being contained within a large diffuse nucleus. In still other stages the nucleus disappears, the chromatic material diffusing out into the cytoplasm, and later, at another stage, to be reorganized and incorporated into spore material. The presence of a nuclear membrane is never much accentuated at any stage.

The changes observed by the writer and outlined above have been attributed to physiologic cyclic changes and, in this case at least, he is unable to decide upon the phylogenetic status of the organism based on the structure of the nucleus and karyosome.

The trophozoites preparatory to division come to rest and assume a more or less round form (prophase). The karyosome then splits into two equal halves (Pl. I, Figs. 7, 8, metaphase). A centriole was never seen. In the next stage or early anaphase (Fig. 9) the nucleus is greatly hypertrophied and contains a spindle with the karyosomic halves diverging. In a late anaphase (Fig. 10) the nucleus has disappeared; an interzonal region shows clearly between the two karyosomic halves and there is a weak suggestion of a modification of the astral ray phenomenon. Figure 11 represents the telophase with the organization of two nuclei containing each a karyosome. This condition is later followed by cell division into two daughter amoebae which separate and move off one from another. The agamic method of reproduction in this amoeba seems not to be preceded or accompanied by any phenomenon of reduction. At this time no purification of the karyosome occurs as recognized by the formation of chromidia in the nucleus. The nucleus during its existence up to the late anaphase remains perfectly clear.

Another method of multiplication is resorted to in this species, namely, through the formation of spore-like structures homologous to those found by Schaudinn in *Entamoeba histolytica,* and by Walker in a number of amoebae parasitic within the intestinal tract. The details of this sporulation process in *A. commutabilis* again differ from the previously described cases. Sporulation occurs rarely on the depression slides (hanging plates), but most frequently in the Petri plate cultures. Indeed, even in such cultures very often the cyst, trophozoite cyst cycle may be all that is revealed and the other stages are entirely omitted. The conditions under which these phenomena occur or not are obscure. Physical factors may be involved or certain processes may occur only after a definite number of agamic reproductive generations. Sporulation, when it does occur within a Petri plate culture, is quite universal within the particular culture, and one has no difficulty in tracing certain détails of the process and some of the interesting ones that follow.

As was pointed out previously, the early cyst assumes the aspect illustrated on Plate I, Fig. 6. The amoeba assumes a round form, with the chromatic material concentrated as a karyosome and submerged within a clear nucleus. The time required for certain changes varies so much that it is impossible to give any specific data on this point. Generally speaking, in about 12 hours the karyosome begins to purify itself by throwing out or secreting chromidia into the nucleus (Fig. 12). On account of the minuteness of the structures involved in the process, it is impossible to affirm with any conviction that the chromidia are ultimately incorporated within the forming spores and that later some of them are also used during the synthesis of the new trophozoite nucleus. However, the process as it was observed is very suggestive of these theoretical assumptions.

About 6 hours after the stage described above the condition represented by fig. 13 is found. The karyosome has vanished or has been entirely resolved into chromidia, which are now represented collectively by a large diffuse nucleus. Still later (Fig. 14) the nucleus disappears and minute, deeply staining chromatic bodies are found scattered through the cytoplasm. Later stages (Figs. 13 to 18) follow rapidly; some of them seem to consume only a few hours. Figure 15 shows the growth of the deeply staining bodies which are the so-colled spores. If this stage is transplanted to fresh media containing living bacteria, the trophozoite emerges from the cyst in about 12 hours. From cyst to trophozoite, when a fresh cyst is transplanted, consumes in all about 48 hours. The newly emerged trophozoite still contains no nucleus; only spores varying in numbers from five to eight (Fig. 16). The spores also vary considerably in size from quite small ones to some approaching the size of the karyosome of this species. At a later stage (Fig. 17) the trophozoite shows a nucleus and karyosome. The spores are now extruded within a few hours. At this period the writer found no large amoebae undergoing cytolysis, so it appears that the adults after spore extrusion continue to live and divide agamically.

The method of sporulation described does not harmonize with Walker's (1908) general statement that "sporulation like other vital processes, ceases with the encystment of the amoebae." Also in no case was Walker able to observe any nuclear changes preceding or during the process of sporulation. Nuclear changes do precede and accompany sporulation in the species here studied, and in this fact we seem to agree more nearly with Schaudinn (1903) who found in the case of *Entamoeba coli* that profound nuclear changes and a sort of sexual process resulting in eight daughter nuclei and in the corresponding number of amoebae accompany the process of encystment. It seems to be unsafe to make a general statement covering all of the manifold and complicated processes occurring within a class of organisms represented by so many species and still so little understood. One set of cytological and other phenomena may be observed in one species, whereas the reverse or a totally different set of processes may become apparent in another.

In *A. commutabilis* the spores soon develop on the Petri plate cultures if conditions are favorable. Often they fail to develop at all, and it is necessary to make many cultures in order to procure a number that are favorable for study. Development seems to be direct (Fig. 18) as Walker found in other species. At first the spore resembles the karyosome of the amoeba in size and structure. Many individuals show a densely staining periphery and a lighter center. In a short time, the spore enlarges and becomes delicately and uniformly granular throughout with a small number of deeply staining granules in the cytoplasm. The next stage assumes a more amoeboid aspect; larger in size with the organization of chromatic material near the center. The final stage in this series is represented by a small amoeba with a well organized nucleus and karyosome. At this time the organism shows amoeboid movement.

The possibility exists that the deeply staining bodies encountered and termed spores may not be spores at all, but deeply staining chromatic material with a small enveloping layer of cytoplasm which the writer was unable to demonstrate due to faulty technic. In comparison with certain cytologic changes occurring in *E. coli* and *E. histolytica,* it is conceivable that the deeply staining bodies really represent aggregations of chromidia which later surround themselves with a small amount of cytoplasm. These are extruded as very minute amoebae, at first showing no amoeboid movement. The parent *A. commutabilis* does not fragment; the young amoebae or spores are extruded. No autogamous processes preceding the formation of these so-called spores was observed.

Another stage in the complicated life cycle of this amoeba was found, and this stage was the one which really attracted the writer to the interesting potentialities of this species. Budding occurs in a large number of cultures under the proper conditions. Agamic simple and multiple budding was observed by Schaudinn in *Entamoeba histolytica.* Schaudinn not only observed this phenomenon in material taken from the intestinal lumen of cases of dysentery, but also observed it between the cells of the intestinal epithelium. Schaudinn considered that amitotic division of the nucleus accompanies this method of multiplication.

In so far as it is possible to determine, Schaudinn seems to have been the only worker who observed budding in amoebae, and when this condition first appeared on the *A. commutabilis* plates a contamination with a saccharomycete or with the yeast-like stages of some of the higher fungi suggested itself. After repeated isolations and platings the writer was able to convince himself that budding occurs at times within the species studied.

Budding when it does occur in *A. commutabilis* appears about 12 to 24 hours after the so-called spores have given rise to small amoebae. The small amoebae at first grow a bit and divide a number of times (Fig. 19). Soon after one begins to recognize such forms as are represented by figure 3 (from living specimen) and figure 20 (fixed and stained). A small bud will be found beginning to constrict off from the parent cell and without the suggestion of any nucleus. The nucleus of the parent cell shows the presence of a karyosome and chromatin granules within the otherwise clear nucleus. These chromatin granules or chromidia undoubtedly diffuse out into the cytoplasm and some of them probably reach the bud before constriction occurs. Figure 21 shows another case of budding with the karyosome in a curious, stellate, cyclic phase. Figure 22 represents a larger amoeba budding and also showing pseudopodial activity. By examining a number of budding cultures in the fresh condition with the aid of the warm stage, the writer has frequently observed budding accompanied by slight and slow pseudopodial activity in certain larger individuals. Generally speaking, however, budding is a function of the small amoebae and usually disappears entirely when they become large. Figure 23 is difficult to interpret. This case may represent unequal division of a small amoeba or rather equal budding or, lastly, it may represent an abnormal condition. Multiple budding, as observed by Schaudinn in *E. histolytica,* does not occur.

After the buds have formed they are gradually constricted off. The time required for this and some of the other processes has not been noted, for the reason that budding does not occur on the hanging plate cultures which lend themselves better to time observations. Figure 24 represents a number of stages in the development of the bud to the small amoeba. The first stage looks much like the adhering bud. It is more or less round and finely granular with no nucleus. Later an organization of chromatic material occurs and the bud begins to assume an amoeboid form. On examining the Petri cultures still later, one finds typical small amoebae (Fig. 25) showing a clear nucleus with the deeply staining karyosome.

The stages represented by figures 26 and 27 are also difficult of interpretation and I hesitate to offer one. These stages were rarely found in resting amoebae, not cysts, and probably represent nuclear cyclic changes. The ray-like structure (Fig. 26) is not the opening in a cyst through which an amoeba has emerged, but a nucleus with peripheral chromatin and sending chromatic rays into the cytoplasm.

Amoeba vegetabilis sp. nov.

This species is an exceedingly active form when examined on the hanging plate cultures. A distinct differentiation between endo and ectoplasm exists (Plate 11, Figs. 28, 29, 30 and 31) except when in the resting condition (Figs. 35 and 36). A continual streaming of the entire protoplasm occurs. The endoplasm is granular and contains many ingested bacteria. The formation of the pseudopodia is not eruptive as in the preceding species, but they form in an even flowing manner. One or two broad pseudopodia occur which may terminate bluntly (Fig. 28) or in a pointed or saw-toothed fashion (Figs. 29 and 30). A contractile vacuole is present, and an interval of approximately 65 seconds intervenes between two pulsations. After contraction the new vacuole arises again at two or three minute points that increase in size and suddenly flow together into one large vacuole which soon contracts. The nucleus is of the karyosomic type with all the chromatin concentrated in the center and submerged in a clear surrounding area. This type of nucleus seems to exist except during the greatest feeding and digestive phases when (Figs. 32 and 33) the chromatic material is diffuse throughout and no karyosome is present. No especial study of the agamic multiplication of this amoeba was made, but since the nucleus is of the karyosomic type a form of mitosis seems probable.

This amoeba was also studied on Petri plate cultures and the vegetative or agamic method of reproduction appeared to be the only one. The formation of spores or a process of budding was never observed. The cyst, trophozoite, cyst cycle, consuming from 5 to 9 days, constituted the entire history on the media used. When cysts are placed on fresh media in the presence of suitable bacteria (*Staphylococcus pyogenes albus*) and kept at room temperature, the trophozoites emerge in from 24 to 48 hours.

When fixed and stained properly, the cytoplasm of the trophozoites appears to be highly vacuolated (Figs. 32, 33, 34 and 35). Large food vacuoles are also in evidence containing phagocytized bacteria. Figure 33 represents the only fixed specimen found showing a large ectoplasmic pseudopodium. Figure 35 represents a round resting stage.

When the amoeba begins to encyst, it comes to rest, becomes round and the cytoplasm loses its vacuolated appearance becoming granular (Fig. 36). No karyosome can be seen, the entire nucleus staining. After about 12 hours or more the amoeba becomes polygonal or irregular, a contraction of the protoplasm occurs and a two walled cyst is formed (Figs. 37, 38 and 39). In fully formed cysts the amoeba proper may appear to be polygonal and rather regular (Figs. 37 and 38) or it may assume a stellate or irregular form with rounded, blunt angles (Fig. 39). At times the encysted amoeba follows closely the outline of the cyst wall proper, which is usually quite irregularly scalloped. The angles of the amoeba do not touch the cyst wall. The cyst wall proper is clear and hyaline. After the emergence of the amoeba the empty cysts appear yellow in color. One nucleus is present within the cyst. The average resting *A. vegetabilis* measures 10 to 13μ. The nucleus measures 2μ and the cysts 10μ.

The amoeba described as *A. vegetabilis* somewhat resembles Walker's *A. intestinalis*. Several diagnostic distinctions exist, but the principal ones deal with the differentiation of endo- and ectoplasm, with locomotion and reproduction. In *A. intestinalis* the ectoplasm is scarcely apparent except in the pseudopodia; in *A. vegetabilis* (Figs. 28, 29 and 30, from living specimens) the ectoplasm appears usually to envelope the entire amoeba. Furthermore, *A. intestinalis* is not very active, whereas *A. vegetabilis* is exceedingly active. *A. intestinalis* forms spheroidal spores 0.5 to 1.5μ, whereas *A. vegetabilis* does not form any spores.

EXPERIMENTAL DATA

Since Tyzzer and Fabyan (1920) were able to produce certain lesions of blackhead in young turkeys, chickens, and pigeons by subcutaneous and intramuscular inoculation of tissue from liver lesions of acute cases, it seemed advisable to inoculate these birds with cultures of Heterakis ova, and also with the two species of amoebae isolated and described in this paper. Following the directions of the above named workers explicitly, young turkeys, 5 to 9 days old chickens, and pigeons were inoculated. Small, white, indurated masses or, in some cases, quite extensive reactions were obtained after a few days in the connective tissue under the skin. In no case, however, were any of the lesions of blackhead reproduced. Sectioned material was studied extensively, and it was found that the amoebae associated in the Heterakis cultures and used for these experiments were not modified to resemble *Histomonas meleagridis* Tyzzer (*Amoeba meleagridis* Smith) after gaining admission to the bird tissues. Usually the amoebae were killed off by the tissues or, when found, resembled the amoebae as they appear in culture.

Whereas it has been proven by Graybill and Smith (1920) that one of the factors for the production of blackhead in turkeys and chickens is present in the Heterakis cultures in the form of the embryonated worms, the results of the experiments here briefly outlined seem to show that the protozoan factor is missing in such cultures.

SUMMARY

1. Commonly one of two species of amoebae or both species of amoebae developed within embryonated Heterakis salt solution cultures. The amoebae were cultivated in pure cultures, and described as two new species, viz., *A. commutabilis* and *A. vegetabilis*.
2. In *A. commutabilis* a modification of the promitotic method of division is described. In this same amoeba interesting nuclear cyclic changes were observed.
3. New cytologic details accompanying and following sporulation were elucidated in *A. commutabilis*. A process of budding was discovered, accompanied and followed by nuclear cyclic changes. A sexual process was not observed.
4. Agamic division, a kind of sporulation, and budding constituted the methods of multiplication in the life history of *A. commutabilis*.
5. Agamic division, probably promitotic, was the only method of multiplication found in *A. vegetabilis*.
6. Turkeys, chickens, and pigeons when inoculated with Heterakis cultures containing one or both species of amoebae, or when inoculated with pure cultures of the two speciaes of amoebae, failed to develop lesions of blackhead.

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Explanation of Plate I

Plate I.—*Amoeba commutabilis*. × 813. Figs. 1-5, Living trophozoites. 6, Cyst. 7-11, Fixed and stained preparations showing promitotic division. 12-18, Process of sporulation. 18, Development of spore. (Fig. 12, fixed and stained. Figs. 13-18, stained intravitally.) 19-24, Fixed and stained preparations showing process of budding. 24, Development of bud. 25, Young trophozoite derived from bud. 26-27, Uninterpreted nuclear cyclic changes.

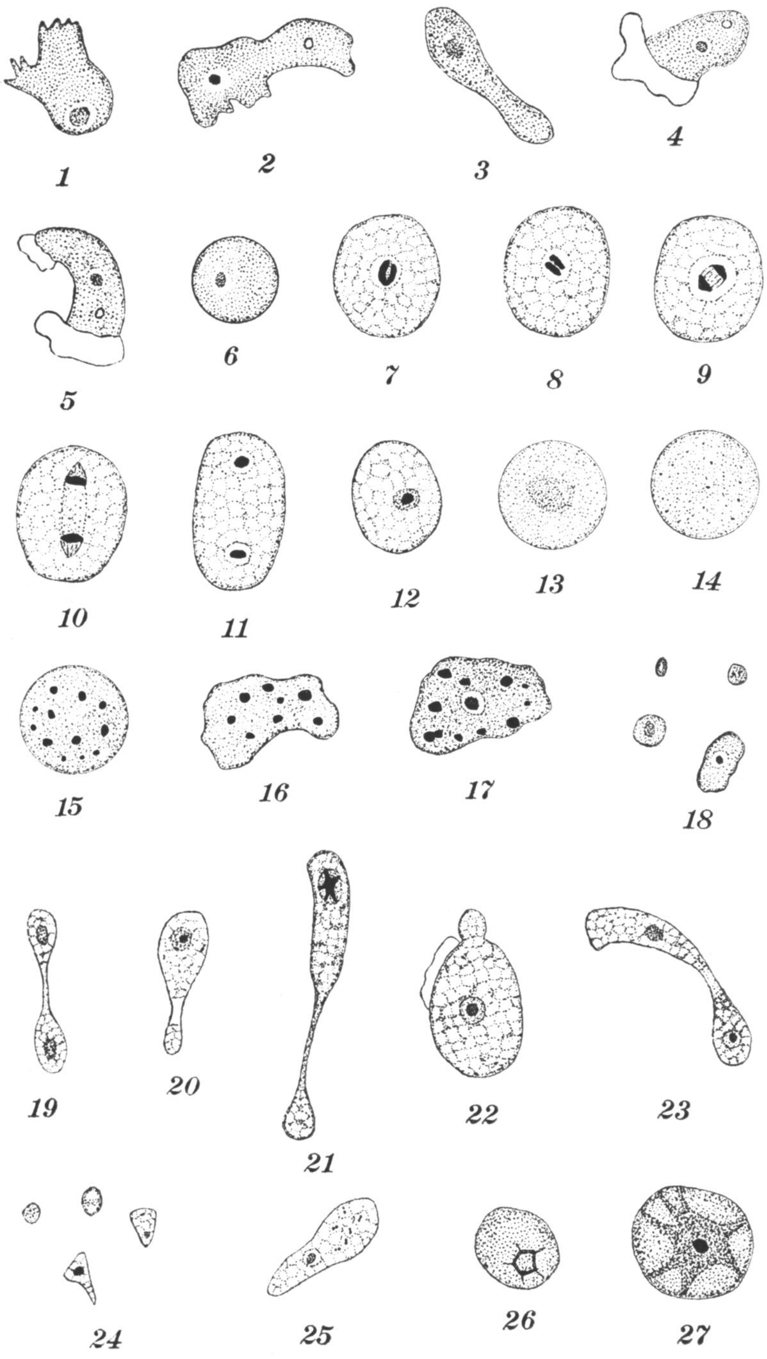

PLATE I

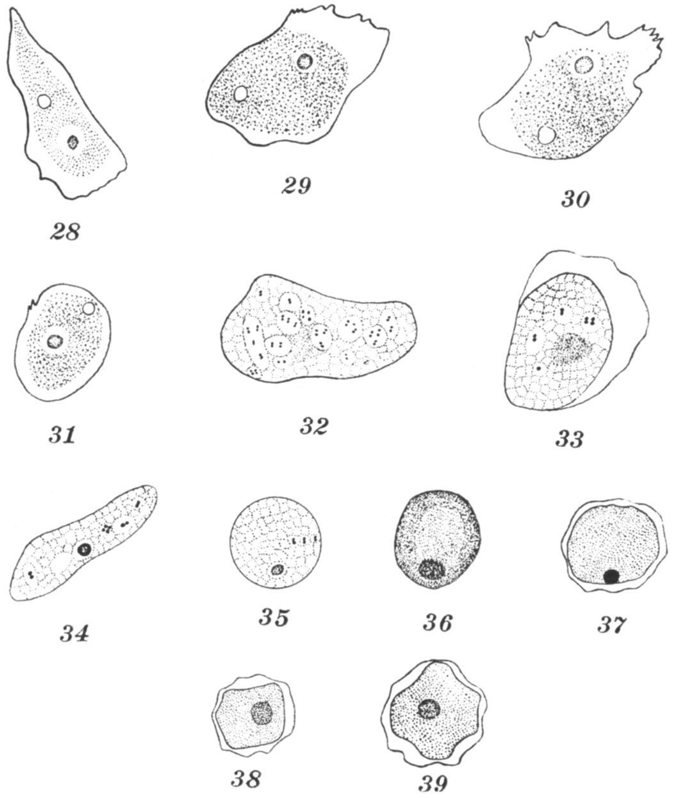

PLATE II

Explanation of Plate II

Plate II.—*A. vegetabilis.* × 813. Figs. 28-31, Living trophozoites. 32-34. Fixed and stained trophozoites. 35, Resting stage. Fixed and stained. 35, Early cyst. 37-38, Types of mature cysts.